

# Elk-1 protein domains required for direct and SRF-assisted DNA-binding

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## ABSTRACT

**The Ets-related Elk-1 protein can bind to purine-rich DNA target sites in a sequence specific fashion and, in addition, can form a ternary complex with the *c-fos* serum response element (SRE) and the serum response factor (SRF). We demonstrate that Elk-1 can readily interchange between its different interaction partners. The amino terminal ETS-domain of Elk-1 was shown to be necessary and sufficient for direct DNA-binding activity. For ternary complex formation with the SRE and SRF, both the Elk-1 ETS-domain as well as flanking sequences up to amino acid 169 were required. Removal of sequences between the ETS-domain and amino acids 137–169 did not abolish ternary complex formation. This suggests the Elk-1 region spanning amino acids 137–169 to contain a protein-protein interaction domain. Furthermore, we have shown that a single amino acid exchange introduced into the ETS-domain can drastically alter the direct DNA-binding affinity of Elk-1 without severely affecting SRF-assisted binding to the SRE. Thus, Elk-1 requires different propensities of the ETS-domain to exert its different modes of DNA sequence recognition.**

## INTRODUCTION

The *ets* proto-oncogenes encode DNA-binding factors that are thought to regulate gene expression by controlling transcription initiation. A variety of members of this gene family have been isolated from vertebrates (man, mouse, chicken) or invertebrates (*Drosophila*): *ets-1* and *ets-2* (1,2), *erg* (3), *elk-1* and *elk-2* (4), *SAP-1* (5), *PU.1* (6), *Fli-1* (7), *GABP $\alpha$*  (8), *E74* (9,10), *D-ets-2* (11) and *D-elg* (12). Through alternative splicing of the *erg*, *SAP-1* and *E74* genes the diversity of the Ets oncoprotein family is further enhanced (3,5,9,10). In addition, a viral counterpart to the *ets-1* gene exists in the avian retrovirus E26 as part of a tripartite oncogene, which is a fusion gene derived from the viral *gag* gene and the chicken *myb* and *ets-1* genes (13,14).

All members of the Ets-protein family are characterized by a homologous ETS-domain of approximately 85 amino acids, but are most often unrelated outside this domain. It has been proposed that the ETS-domain represents a novel DNA-binding

motif that recognizes purine-rich target sequences (15). Deletion studies on the Ets-1 (16), the PU.1 (6) and the GABP $\alpha$  protein (17), in which the ETS-domain was shown to be necessary for the DNA-binding activity, support this hypothesis. Furthermore, a truncated GABP $\alpha$  protein, which is 94 amino acids long and encompasses the ETS-domain, is capable of binding specifically to DNA (17). As this truncated GABP $\alpha$  protein is only slightly larger than the ETS-domain, it implies that the ETS-domain may not only be necessary, but also sufficient for DNA-binding activity.

*In vivo* transcriptional activation exerted by Ets proteins was shown for PU.1, Ets-1, Ets-2, Erg-1, Erg-2 and Elk-1 in cotransfection experiments using reporter constructs driven by corresponding binding sites (6,18–21). Interestingly, Ets-1 and the AP1 transcription factor were able to cooperate for transcriptional activation when utilizing the oncogene-responsive domain of the polyoma enhancer as promoter element of the reporter construct (18). Cooperativity with other transcription factors might also play an important role for GABP $\alpha$  and the E74 proteins. The former binds only efficiently to target DNA sequences upon interaction with GABP $\beta$  (17), while the latter ones are thought to regulate an ecdysteroid induced puffing sequence in *Drosophila* together with other transcription factors (22), the ecdysteroid receptor (23) and the E75 proteins (24,25).

Recently, Elk-1 was demonstrated *in vitro* to behave indistinguishably from p62<sup>TCF</sup> (26), a protein capable of binding to the serum response element (SRE) of the *c-fos* promoter in a complex with the serum response factor (SRF). Like p62<sup>TCF</sup> Elk-1 does not bind directly to the SRE (27,28). No difference was detected in band-shift or methylation interference assays in the way p62<sup>TCF</sup> and Elk-1 form a ternary complex with the SRF and SRE. Furthermore, p62<sup>TCF</sup> and Elk-1 are antigenically related, which hints at a high degree of homology, if not identity, between these two proteins. Since p62<sup>TCF</sup> is apparently important for the proper induction of the *c-fos* gene (27,29), Elk-1 could also be involved in the regulation of the proto-oncogene *c-fos*.

In this study we characterized the Elk-1 protein by investigating its direct DNA-binding to a purine-rich target sequence as well as its SRF-assisted binding to the *c-fos* SRE. N- and C-terminal truncations of Elk-1 allowed the mapping of domains necessary to perform these functions. Our results further validate the

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hypothesis that the ETS-domain is a DNA-binding entity and hint at a new protein-protein interaction domain that directs the interaction between Elk-1 and SRF.

## MATERIALS AND METHODS

### Construction of Elk-1 variants

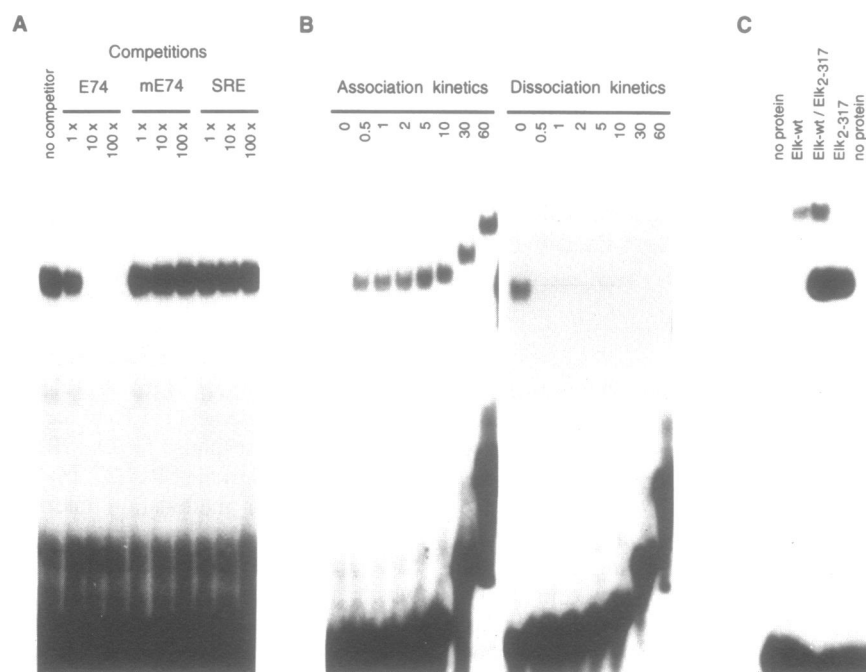
Recombinant DNA manipulations were performed according to standard procedures (30). DNA sequences generated with the help of the polymerase chain reaction were confirmed by sequencing. All Elk-1 constructs were cloned into the eukaryotic expression vectors pEVRF0-2 or pEV3S (31). Elk $_{\Delta 1-33}$  and Elk $_{\Delta 1-82}$  consisted of the peptide MASWGSGTQ fused to amino acids 34–428 of Elk-1 or of the peptide MASWGSGYP fused to amino acids 83–428 of Elk-1, respectively. In the other Elk-1 variants, including Elk-wt, the first methionine was replaced by MASWGS. C-terminal deletions possessed at their carboxy terminus the following 2–11 amino acids derived from vector sequences: Elk $_{2-82}$  (WVPS), Elk $_{2-93}$  (GT), Elk $_{2-119}$  (GYLAS), Elk $_{2-152}$  (WVPS), Elk $_{2-157}$  (YLAS), Elk $_{2-169}$  (GGSELPGYLAS), Elk $_{2-187}$  (ARGIRAPRVPS), Elk $_{2-215}$  (RYLAS), Elk $_{2-287}$  (GYLAS), Elk $_{2-317}$  (VPS) and Elk $_{2-374}$  (GYLAS). Internal deletions were identical to Elk $_{2-169}$ , except that amino acids 94–119 (Elk $_{2-93/BI}$ ) or 94–136 (Elk $_{2-93/BS}$ ) were replaced by the dipeptide GT or that amino acids 83–119 (Elk $_{2-82/BI}$ ) or 83–136 (Elk $_{2-82/BS}$ ) were replaced by the tetrapeptide WIGT. Elk-D74 was derived from Elk-wt by changing arginine at position 74 to aspartic acid.

### Expression of proteins in HeLa cells

HeLa cells, which were grown on a 10 cm dish in DMEM + 10% fetal calf serum to 50% confluency, were transfected with 30  $\mu$ g of recombinant expression vector using the calcium-phosphate coprecipitation method (30). The precipitate was removed after 6 h and the cells were further grown in DMEM + 10% fetal calf serum for 12–18 h. Cells were then washed twice with PBS, detached from the dish by incubation in 40 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA for 5 min and collected by centrifugation (1,000 $\times$ g, 3 min). The cell pellet was resuspended in 0.5 ml of 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl $_2$ , 10 mM NaF, 0.5% Triton X-100, 0.05% sodiumdeoxycholate, 1 mM DTT, 0.5 mM PMSF and lysis was allowed to proceed for 20 min on ice. Debris were removed by centrifugation (10,000 $\times$ g, 10 min, 4°C). The supernatant, which contained the expressed Elk-1 variants to be used in gel retardation assays, was frozen in liquid N $_2$  and stored at –80°C.

### Gel retardation assays

Binding reactions were performed at room temperature for 30–90 min and contained 6.5  $\mu$ l buffer A (10 mM Hepes pH 7.9, 5 mM MgCl $_2$ , 0.1 mM EDTA, 10 mM NaCl), 1  $\mu$ l 10 $\times$ SED (20 mM spermidine, 25 mM EDTA, 133 mM DTT), 1  $\mu$ l of a 2 mg/ml poly(dIdC):poly(dIdC) solution, 1  $\mu$ l of 20 mg/ml BSA and 0.1  $\mu$ l  $^{32}$ P labeled oligonucleotide (0.5–1 ng). This was supplemented with up to 2  $\mu$ l of protein extracts or purified proteins. For loading on a 4% polyacrylamide/0.5 $\times$ TBE



**Figure 1.** Binding of Elk-1 to the E74 binding site as assayed by gel retardation. (A) Complex formation between Elk-wt and  $^{32}$ P labeled E74-oligo. Competitor oligo (E74, mE74 or SRE) was added to preformed complex in the indicated molar excess and reactions were allowed to proceed for another 30 min. (B) Kinetic studies on the interaction between Elk-wt and  $^{32}$ P labeled E74-oligo. For the association kinetics numbers indicate the times in minutes after the addition of Elk-wt to the binding reaction. For the dissociation kinetics, numbers indicate the times in minutes after the addition of a 20-fold excess of unlabeled E74-oligo to the preformed complex. Samples were applied onto a continuously running gel. (C) Expression vectors encoding Elk-wt or the carboxy terminal truncation Elk $_{2-317}$  were either separately or jointly transfected into HeLa cells. Complexes formed by the corresponding protein extracts with the  $^{32}$ P labeled E74-oligo are depicted; no protein extract was added in the outer two lanes.

gel, samples were mixed with 2  $\mu$ l of 10% Ficoll 400, 15% glycerol, 40 mM EDTA. Electrophoresis was performed at room temperature in 0.5 $\times$ TBE. Gels were dried and protein-DNA complexes were revealed by autoradiography at  $-80^{\circ}\text{C}$ .

The used SRF was affinity purified SRF-6His expressed in vaccinia virus-infected HeLa cells (32), while the SRF- $\Delta$ 245, which was his-tagged at the amino terminus and affinity purified by metal chelate chromatography as SRF-6His, was derived from transiently transfected HeLa cells. SRF-Core was generated by *in vitro* transcription/translation and was kindly provided by R.A. Hipskind. Double-stranded  $^{32}\text{P}$  labeled oligos were generated by hybridization of complementary oligonucleotides and subsequent filling in of 3'-recessed termini with the Klenow fragment of DNA polymerase I in the presence of  $\alpha$ - $^{32}\text{P}$ -dATP (30). The double-stranded SRE-oligo spanned the human *c-fos* promoter from  $-324$  to  $-300$  and the sequence of the double-stranded E74-oligo was the following: 5'-AGCTTCTCTAGCTGAATAA-CCG\*G\*AAGTAACTCATCGTCA-3'.

The mE74-oligo differed from this sequence by two exchanges: the two guanine residues marked by asterisks were replaced by two cytosine residues.

## RESULTS

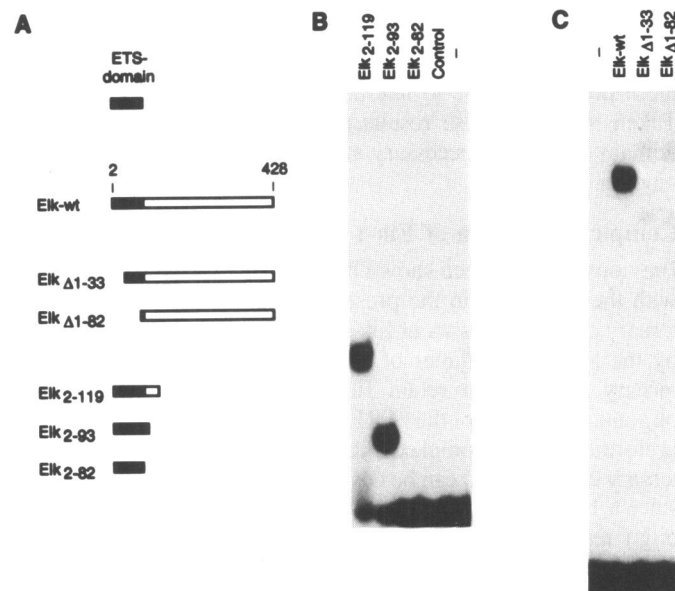
### Direct DNA-binding of Elk-1

Bacterially expressed Elk-1 protein has been shown to bind specifically to several DNA sequences. The highest affinity was observed for an E74 binding site (E74-BS), which is a natural target sequence of the *Drosophila* Ets-related E74 proteins (21,33). We chose this sequence to investigate the direct DNA-binding properties of Elk-1. To obtain Elk-1 protein in high

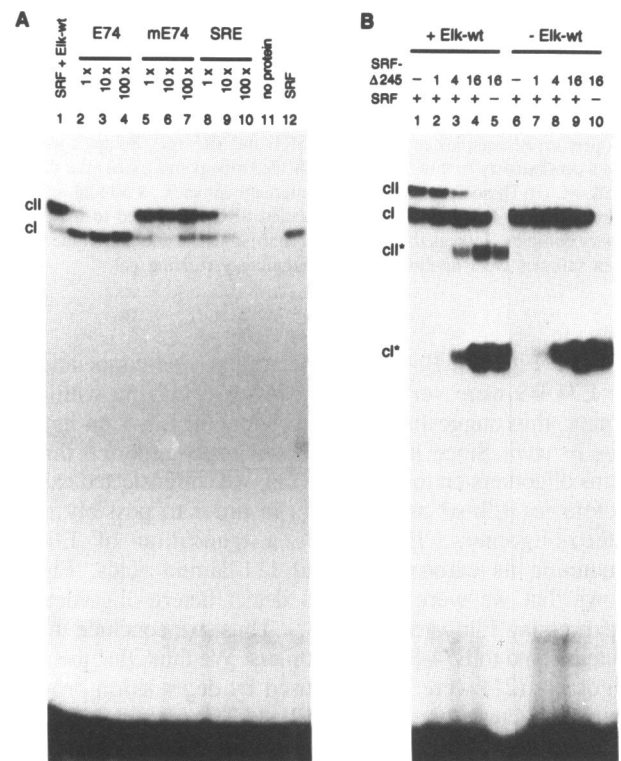
amounts and in a post-translationally modified form, we cloned *elk-1* cDNA into a eukaryotic expression vector and derived corresponding Elk-wt protein from transiently transfected HeLa cells. The Elk-wt protein contained all of the 428 amino acids of Elk-1 with the exception of the first methionine, which was replaced by the peptide MASWGS.

In gel retardation assays the Elk-wt protein formed a complex with the E74-BS (Fig. 1A). Upon shorter exposition of the autoradiogram (not shown) this complex was resolved into two differently migrating components. This result suggests that the Elk-wt protein used was heterogeneous, which might be due to post-translational modifications such as phosphorylation. The specificity of complex formation between the Elk-wt protein and the E74-BS was demonstrated by competitions with unlabeled oligos (Fig. 1A) containing the E74-BS (E74-oligo), a mutated E74-BS (mE74-oligo) unable of interacting with Elk-1 (21) or the SRE, to which Elk-1 binds only in the presence of SRF (26). A 10-fold excess of the unlabeled E74-oligo was sufficient to suppress any detectable binding to the  $^{32}\text{P}$  labeled E74-BS, while even a 100-fold excess of mE74-oligo or SRE-oligo did not compete the binding to the E74-BS.

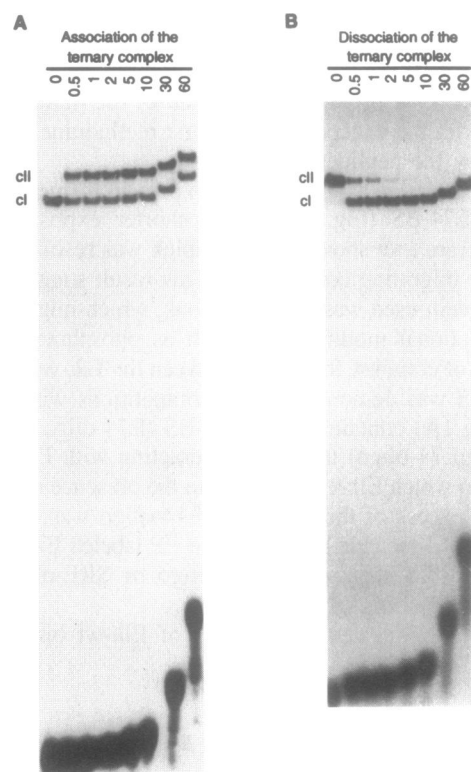
We next investigated the kinetics of Elk-wt binding to the



**Figure 2.** Requirement for the ETS-domain of Elk-1 for direct DNA-binding. (A) Schematic presentation of amino and carboxy terminal deletions. (B) Gel retardation assay with carboxy terminal Elk-1 truncations and  $^{32}\text{P}$  labeled E74-oligo. Control denotes a protein extract derived from HeLa cells transfected with the expression vector pEV3S. A dash indicates that no HeLa protein extract was added to the binding reaction. (C) Analogous for Elk-1 amino terminal truncations.



**Figure 3.** (A) Elk-1 interaction with the SRE:SRF complex: formation of the ternary complex cII revealed by gel retardation. Lanes 1–10: SRF and Elk-wt were allowed to form a ternary complex with  $^{32}\text{P}$  labeled SRE-oligo. Competitor oligo (E74, mE74 or SRE) was added, followed by 15 min of incubation. The molar excess of the competitor over the  $^{32}\text{P}$  labeled SRE is indicated. As a control, the labeled SRE was incubated without SRF and Elk-wt (lane 11) or with SRF alone (lane 12). cI: binary complex formed between the SRE and SRF. (B) Preformed ternary complex of  $^{32}\text{P}$  labeled SRE, SRF and Elk-wt (cII, lanes 1–4) was supplemented with increasing amounts of preformed binary complex of labeled SRE and SRF- $\Delta$ 245 (cI\*). Relative amounts of total SRF- $\Delta$ 245 are indicated. cII\* denotes the ternary complex containing SRF- $\Delta$ 245. No SRF was present in lane 5. Lanes 6–10 correspond to lanes 1–5, but no Elk-wt was present.



**Figure 4.** (A) Association kinetics of the ternary complex (cII). Elk-wt was added to a preformed complex of  $^{32}\text{P}$  labeled SRE and SRF (cI). Samples were loaded onto a continuously running gel at the indicated time points (min) after the addition of Elk-wt. (B) Dissociation kinetics of the ternary complex. A 50-fold molar excess of unlabeled E74-oligo over the  $^{32}\text{P}$  labeled SRE was added to the preformed ternary complex. Indicated are the times (min) after the addition of the E74-oligo when samples were applied onto a continuously running gel.

E74-BS (Fig.1B). Both the association to and dissociation from the E74-BS were very rapid processes occurring within half a minute, thus suggesting a fast exchange of Elk-1 on its binding sites *in vivo*. Since it has been previously reported that Elk-1 forms oligomers on the E74-BS (21), we cotransfected expression vectors for Elk-wt and Elk<sub>2-317</sub> in order to possibly generate hetero-oligomers. Elk<sub>2-317</sub> is a truncation of Elk-1 not containing its carboxy terminal 111 amino acids. Figure 1C shows that we were unable to detect hetero-oligomers when coexpressing Elk-wt and Elk<sub>2-317</sub>. Thus, we conclude that Elk-1 proteins bind to E74-BS as monomers. We think that the reported oligomers (21) were in fact caused by degradation products of the used bacterially expressed Elk-1. The pattern of interaction of bacterially expressed Elk-1 with the SRE:SRF (26) is also consistent with the presence of Elk-1 breakdown products.

Amino acids 3–86 of Elk-1 comprise the ETS-domain. This domain was proposed to confer the DNA-binding activity to all members of the Ets family (15). To validate this proposition carboxy terminal deletions of Elk-1 were assayed for their capability to bind to E74-BS (Fig.2A,B). Deletion of 309 or 335 amino acids (Elk<sub>2-119</sub> and Elk<sub>2-93</sub>, respectively) did not abolish the DNA-binding activity, while deletion of 346 carboxy terminal amino acids (Elk<sub>2-82</sub>) resulted in a loss of DNA-binding activity. This delimits a carboxy terminal boundary of the Elk-1 DNA-binding domain within amino acids 82–93 that may be identical to the carboxy terminal boundary of the Elk-1 ETS-

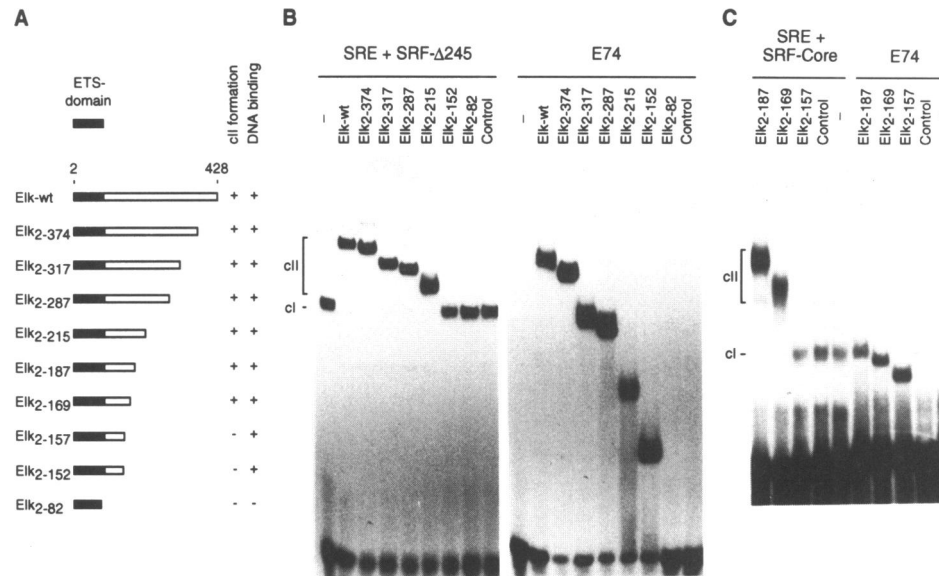


**Figure 5.** Stoichiometry of the ternary complex. Two carboxy terminal truncations of Elk-1 (Elk<sub>2-215</sub> and Elk<sub>2-374</sub>) were either expressed alone or together in HeLa cells. Corresponding protein extracts were used to examine the interaction with the  $^{32}\text{P}$  labeled SRE-oligo and SRF-Δ245 by gel retardation assays. Lane 2 contained Elk<sub>2-374</sub>, lane 3 coexpressed Elk<sub>2-374</sub> and Elk<sub>2-215</sub> and lane 4 Elk<sub>2-215</sub>, while lanes 1 and 5 contained no Elk-1 variant. cI: binary complex; cIIa and cIIb: ternary complexes formed by Elk<sub>2-374</sub> and Elk<sub>2-215</sub>, respectively; cIIa: higher order complex.

domain at amino acid 86. Furthermore, deletion of the first 82 (Elk<sub>Δ1-82</sub>) or 33 (Elk<sub>Δ1-33</sub>) amino acids of Elk-1, that respectively eliminates 95% or 40% of the ETS-domain, rendered these proteins unable to interact with the E74-BS (Fig.2A,C). Taken together, these results provide evidence that the ETS-domain of Elk-1 is necessary and sufficient for its direct DNA-binding activity.

#### Complex formation of Elk-1 and the SRE:SRF

Previously, it has been shown that the Elk-1 protein can interact with the SRE only in the presence of SRF (26). The resulting ternary complex consists of Elk-1 and a binary complex generated by the SRE and a dimer of SRF. We tested the ability of the ternary complex to retain Elk-1 by adding either different oligonucleotides or the SRE:SRF binary complex to the preformed ternary complex. Figure 3A shows that the preformed ternary complex (cII) can be destroyed by the addition of E74-BS, since a 10-fold molar excess of the E74-oligo over the used SRE-oligo resulted in the appearance of only the binary complex SRE:SRF (cI). As a control, the mutated E74-BS (lanes 5–7) did not lead to the destruction of the ternary complex, while addition of unlabeled SRE-oligo (lanes 8–10) led to both the destruction of the binary and ternary complexes. We next assessed the ability of preformed binary complex to compete with preformed ternary complex for Elk-wt protein. For this, a ternary complex of SRF, SRE and Elk-wt was preformed and then a binary complex consisting of the SRE and a truncated version of SRF (SRF-Δ245), that contains SRF amino acids 10–245, was added. Figure 3B shows that with increasing amounts of the

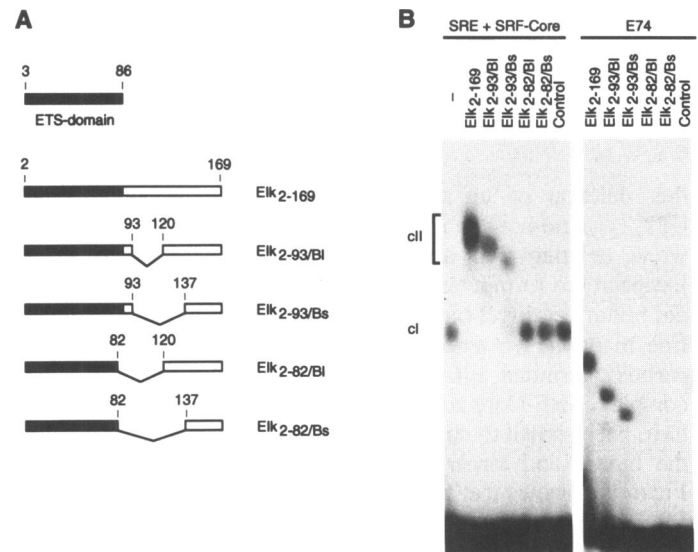


**Figure 6.** (A) Schematic outline of Elk-1 carboxy terminal truncations. (B) The different Elk-1 variants were assessed by gel retardation assays for their ability to form the ternary complex (cII) together with a binary complex (cI), that contains  $^{32}$ P labeled SRE-oligo and SRF-Δ245, and also for their ability to interact directly with the E74 binding site. Control denotes a protein extract derived from HeLa cells transfected with the eukaryotic expression vector pEV3S and a dash indicates that no protein extract was added. (C) Analogous, except that SRF-Core was used instead of SRF-Δ245.

binary complex SRE:SRF-Δ245 (cI\*) the ternary complex SRE:SRF:Elk-wt (cII) disappeared and a new ternary complex SRE:SRF-Δ245:Elk-wt (cII\*) was formed. Elk-wt thus apparently interchanges readily between different ternary complexes. Note that due to the stability of SRF dimers no heterodimers were formed when SRF and SRF-Δ245 were mixed (Fig.3B, lanes 7–9) in agreement with previous results demonstrating that heterodimerization requires coexpression (34).

The exchange of Elk-wt between different binary complexes is reflected by the rapid association and dissociation kinetics of the ternary complex (Fig.4): association of Elk-wt with the SRE:SRF complex was nearly complete within half a minute and dissociation of the ternary complex after the addition of the E74-oligo was completed after approximately two minutes. Upon adding an excess of SRE-oligo to the preformed ternary complex, destruction of this complex was complete within 5 minutes which was indistinguishable from the response of the binary SRE:SRF complex to the SRE-oligo competitor (data not shown). This implies that Elk-1 binding to the SRE:SRF complex does not increase the strength of interaction between the SRE and the SRF.

To decipher the stoichiometry of the ternary complex we coexpressed two carboxy terminal truncations of Elk-1 in HeLa cells. Both truncations were able to form a ternary complex (Fig.5, lanes 2 and 4). No intermediate ternary complex was generated when the coexpressed, differently truncated versions of Elk-1 were mixed with SRE:SRF-Δ245 (lane 3). Therefore, the ternary complex must contain only one molecule of Elk-1 because otherwise ternary complexes of intermediate size would have formed. Thus, the ternary complex consists of three components: one molecule of Elk-1, a dimer of SRF and the SRE. This conclusion is consistent with the previous suggestion on the stoichiometry of the ternary complex formed by the p62<sup>TCF</sup> molecule and SRE:SRF (28). We also noticed that the addition of an excess of Elk-1 variants to the SRE:SRF complex led to the formation of higher order complexes as shown for Elk<sub>2-374</sub>

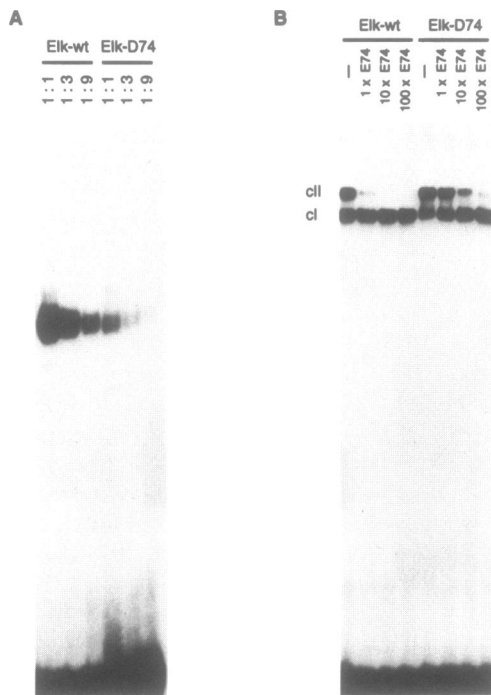


**Figure 7.** (A) Elk-1 internal deletions. (B) Binding of Elk-1 internal deletions to the SRE:SRF-Core complex and to the E74-BS as assayed by gel retardation experiments. Nomenclature as in Fig.6.

(cIIla; Fig.5, lane 2). The relative mobility of cIIla in comparison to the respective ternary and binary complexes is suggestive of an aggregate consisting of two molecules of Elk<sub>2-374</sub> and the SRE:SRF complex.

#### Determination of Elk-1 domains necessary for the interaction with the SRE:SRF complex

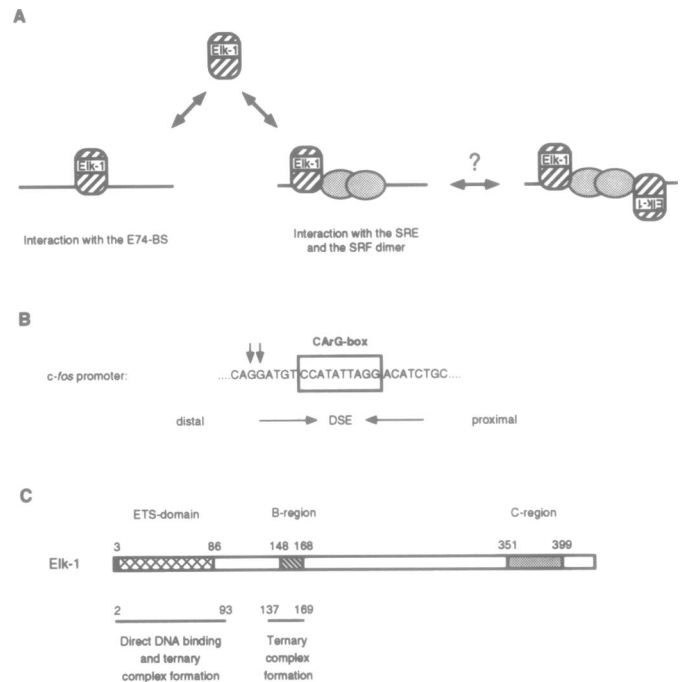
In order to determine the Elk-1 amino acids necessary for complex formation with SRE:SRF, we successively deleted carboxy terminal portions of Elk-1. A coarse mapping indicated



**Figure 8.** Comparison of Elk-wt and the mutant Elk-D74. (A) Formation of a complex with  $^{32}\text{P}$  labeled E74-oligo. Indicated are the employed dilutions of protein extracts. (B) Formation of the ternary complex (cII) with a complex of  $^{32}\text{P}$  labeled SRE and SRF. A 1:10 dilution of both Elk-wt and Elk-D74 was employed. Unlabeled E74-oligo was added to the preformed ternary complex and the incubation went on for another 30 min. Equimolar, 10-fold or 100-fold molar amounts of E74-oligo compared to the  $^{32}\text{P}$  labeled SRE were employed.

that deletion of up to 213 carboxy terminal amino acids (Elk<sub>2-215</sub>) did not abolish the formation of the ternary complex, while deleting 276 amino acids (Elk<sub>2-152</sub>) resulted in an incapacity to form a ternary complex with SRE:SRF-Δ245, but did not affect direct binding to the E74-BS (Fig.6A,B). For the fine mapping we assessed the interaction of three additional carboxy terminal Elk-1 truncations with the SRE:SRF-Core complex. SRF-Core consists of SRF amino acids 133–222 that have been shown to constitute the minimal region necessary for the binary and ternary complex formation of SRF (34,35). Figure 6C shows that Elk<sub>2-169</sub> is still capable of interacting with SRE:SRF-Core while Elk<sub>2-157</sub> is not. This delimits one boundary of Elk-1 necessary for ternary complex formation between amino acid 157 and 169. On the other hand, truncation of even the first 33 amino acids of Elk-1 rendered the protein incapable of interacting with the binary complex (data not shown). The ETS-domain thus appears to be one necessary component for the interaction with the SRE:SRF complex.

We next addressed the question which Elk-1 amino acids between the ETS-domain and amino acid 169 are required for ternary complex formation. Therefore, internal deletions were generated by fusing amino acids 120–169 (suffix 'B1') or 137–169 (suffix 'Bs') to Elk<sub>2-93</sub> or Elk<sub>2-82</sub> (Fig.7A). Testing their ability to interact with the SRE:SRF-Core complex revealed that amino acids 120–169 as well as amino acids 137–169 were sufficient for ternary complex formation when fused to amino acids 2–93 (Fig.7B). However, when fused to amino acids 2–82, neither region 137–169 nor region 120–169 were able to confer ternary complex forming activity to the respective



**Figure 9.** (A) Architecture of Elk-1 containing complexes. (B) Partial sequence of the *c-fos* SRE. Horizontal arrows delimit the dyad symmetry element (DSE), the center of which is a CArG-box. Elk-1/p62<sup>TCF</sup> contact points with the SRE as determined by methylation interference experiments are indicated by vertical arrows. (C) Schematic drawing of the 428 amino acids of Elk-1. The B- and C-region denote homologous regions in SAP-1 and Elk-1.

mutants Elk<sub>2-82/Bs</sub> or Elk<sub>2-82/B1</sub>. This result could have been expected because Elk<sub>2-82</sub> has in contrast to Elk<sub>2-93</sub> a nonfunctional ETS-domain. Accordingly, Elk<sub>2-82/Bs</sub> and Elk<sub>2-82/B1</sub> were unable to bind to the E74-BS, while both Elk<sub>2-93/Bs</sub> and Elk<sub>2-93/B1</sub> did so (Fig.7B). Our findings indicate that interaction of Elk-1 with the SRE:SRF complex is dependent on both the ETS-domain and amino acids 137–169.

#### Alteration of direct DNA-binding affinity without affecting ternary complex formation

We wondered whether the direct DNA-binding affinity of Elk-1 could be altered without affecting the affinity for the SRE:SRF complex. Previously it has been shown that a single point mutation in the ETS-domain of the *v-ets* oncogene affects the differentiation potential of cells infected by the avian leukemia virus E26 (36). It was tempting to speculate that the altered properties of the mutant virus are caused by an altered affinity for target sequences and that this alteration could also be induced by an analogous mutation of the Elk-1 protein. Therefore, we changed amino acid 74 of Elk-1 from arginine to aspartic acid, generating Elk-D74, since the analogous mutation in the *v-ets* oncoprotein was histidine to aspartic acid. A titration of comparable amounts of Elk-wt and Elk-D74, as determined by Western blotting (data not shown), showed that the affinity of Elk-D74 to the E74-BS is approximately 10-fold reduced as compared to Elk-wt (Fig.8A). On the contrary, the affinity for the SRE:SRF complex was not reduced since comparable amounts of both proteins resulted in the same degree of ternary complex formation (Fig.8B). In agreement, an at least 10-fold higher excess of E74-oligo was necessary to dissociate ternary complexes formed with Elk-D74 than with Elk-wt (Fig.8B). The



binding affinity towards the E74-BS thus does not seem to determine the affinity towards the SRE:SRF complex.

## DISCUSSION

The Elk-1 protein can interact with DNA in two different manners (Fig. 9A): either it binds autonomously to a target sequence, e.g. the E74-BS (21), or it binds to the *c-fos* SRE in which case it must be assisted by the SRF dimer (26). Our results have shown that Elk-1 can readily interchange between the E74-BS and the SRE:SRF complex since kinetic processes governing the association to or dissociation from either of both targets are quite fast. Some biological implications may be deduced from the rapid kinetics: the fast association of Elk-1 would allow this protein to exert its function rapidly upon binding, while the fast dissociation would allow it to be involved in transient processes. These characteristics would make Elk-1 suitable for the control of the *c-fos* gene via interaction with the SRE:SRF complex, because the *c-fos* gene is an immediate early gene being rapidly and transiently induced (37). Since a ternary complex factor is occupying the Elk-1 interaction site within the *c-fos* SRE throughout the activation cycle *in vivo* (38), rapid exchange with other ternary complex factors may be occurring. The SAP-1 proteins could be candidates for such a ternary complex factor because they bind to the SRE:SRF complex in a fashion similar to Elk-1 or p62<sup>TCF</sup> (5).

We have shown that the Elk-1 protein binds to the SRE:SRF complex as a monomer, although at a high protein concentration, unlikely to occur *in vivo*, two Elk-1 molecules might be interacting with the SRE:SRF complex (Fig. 9A). Methylation interference studies have shown that Elk-1 or p62<sup>TCF</sup> contact only nucleotides 5' to the SRF-binding site (26,27). How could this asymmetric binding of Elk-1 to the SRE:SRF complex be explained? From methylation interference studies it was deduced that the SRF dimer alone contacts the SRE within the dyad symmetry element (DSE, Fig. 9B). The observed contact pattern as well as the nearly perfect palindromic structure of the DSE suggest that the SRF dimer presents the surfaces of its monomeric subunits in a sterically symmetric manner, each subunit potentially capable of interacting with an Elk-1 molecule. Thus, if protein-protein interactions were exclusively responsible for the binding of Elk-1 to the SRE:SRF complex, one would expect two molecules of Elk-1 to participate in ternary complex formation. Methylation interference and protection studies (5,26,27), as well as mutations in the GGAT-sequence 5' to the DSE (26,27,29), demonstrated that specific contacts of Elk-1 or other ternary complex factors are indispensable for the formation of the ternary complex over the SRE. No GGAT-sequence is present on the 3' side of the DSE (Fig. 9B) providing an explanation for the asymmetric binding of Elk-1 to the 5' side of the SRE:SRF complex. It is interesting to note that another Ets protein (Ets-1) may bind directly to sequences containing a GGAT-core, albeit with a lower affinity as compared to sequences with a GG-AA-core (39) that are suspected to be important for the DNA-binding of Ets proteins (15,21). One may imagine that Elk-1 is binding weakly to the GGAT-sequence and only concomitant protein-protein interaction between Elk-1 and SRF leads to a stable interaction between Elk-1 and the SRE. This would explain why direct binding of Elk-1 to the SRE cannot be observed.

The Elk-1 ETS-domain is absolutely required for the direct interaction with DNA, because upon deleting parts of the ETS-domain we were unable to detect any interaction with the E74-BS.

Furthermore, the Elk-1 variant Elk<sub>2-93</sub>, that is nearly identical to the ETS-domain spanning amino acids 3–86, interacted with the E74-BS. This result strengthens the hypothesis that the ETS-domain is the DNA-binding domain and a functional entity in all Ets proteins (15). The ETS-domain is furthermore indispensable for ternary complex formation. Since a single amino acid substitution in Elk-D74 exerted a negligible effect on ternary complex formation while drastically lowering the capacity to bind to the E74-BS, one possible corollary of these findings is that different parts/amino acids of the Elk-1 ETS-domain may be involved in the interaction with the E74-BS and the SRE:SRF.

In addition to the Elk-1 ETS-domain, approximately 80 flanking amino acids up to position 169 are required for the interaction with the SRE:SRF complex. This resembles the interaction between GABP $\alpha$  and GABP $\beta$  (17) or between SAP-1 and SRF (5), because 37 amino acids of GABP $\alpha$  or 71 amino acids of SAP-1 are also additionally required besides the ETS-domain. Not all of the flanking 80 amino acids are necessary for ternary complex formation, since the deletion of amino acids 94–136 in Elk<sub>2-93/BS</sub> did not abolish ternary complex formation. This dispensability of the deleted amino acids indicates that they represent a flexible hinge between the ETS-domain and that amino acids 94–136 are not directly participating in the complex formation with the SRE:SRF. Interestingly, amino acids 137–169 encompass the B-region recently defined by Dalton and Treisman (5) that is present in both Elk-1 and SAP-1 (Fig. 9C). Since the B-region of SAP-1 was also found to be required for SAP-1 interaction with the SRE:SRF complex, it might represent a novel protein-protein interaction domain. Investigation of other members of the Ets oncoprotein family has also revealed putative protein-protein interaction domains: the above mentioned interaction of GABP $\alpha$  with the ankyrin repeats of GABP $\beta$  (17) and the likely interaction of the E74 proteins with one of the E75 proteins, that might involve the formation of a 'histidine-metal zipper' (40). In general, protein-protein interaction might thus be a common theme among the DNA-binding Ets proteins.

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